Anticoagulant therapy in critical organ ischaemia/reperfusion injury

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Summary
Ischaemia/reperfusion (I/R) injury is central to a number of pathologies including myocardial infarction and stroke. Several cellular processes are involved in the progress of I/R injury, involving complex interactions between coagulation and inflammatory or apoptotic processes. Besides their anti-coagulant function, anticoagulant proteins such as activated protein C (APC), active site inhibited factor VIIa (ASIS), tissue factor pathway inhibitor (TFPI), and antithrombin (AT) are also known for their anti-inflammatory or cell protective effects. This review gives an overview of the application of these anti-coagulants in several animal models of I/R injury in critical organs and describes the effects of these proteins on cellular processes including inflammation and apoptosis. The future testing of mutant forms of some of these inhibitors including APC in a clinical setting should be actively explored.

Keywords
Ischaemia/reperfusion injury, anti-coagulant therapy, animal models, inflammation, apoptosis

Ischaemia/reperfusion injury

According to the World Health Organisation (WHO), cardiovascular disease (CVD) is the most common cause of death worldwide as in 2005, 17.5 million people died from cardiovascular disease, which represents 30% of all deaths. Forty-three percent of these deaths are caused by myocardial infarction accounting for the greatest number of deaths due to CVD.

Myocardial ischaemia is caused by rupture of an atherosclerotic lesion in one or more coronary arteries and the subsequent exposure of its content to the bloodstream leading to formation of a thrombus. This process appears to occur repeatedly prior to myocardial infarction, due to the fact that thrombotic occlusion is oftentimes followed by reperfusion due to clot lysis or embolisation. The process of reperfusion, required to restore blood flow in the ischaemic area is associated with a broad range of pathologies including myocardial stunning, ventricular arrhythmias, reversible micro-vascular injury, and irreversible cell damage that may in fact contribute to (irreversible) damage of the affected organ (1). In general, induction of ischaemia leads to depletion of high energy phosphates eventually resulting in ultra-structural changes such as accumulation of water and electrolytes leading to cell swelling and disruption of cellular organelles (2). This irreversible process of cell death initiates an acute inflammatory response within and around the area of infarction (3). Reperfusion is associated with the generation of reactive oxygen species (ROS) further triggering the inflammatory response through induction of cytokines, chemokines, and expression of adhesion molecules via the nuclear factor-kappa B (NF-kB) pathway (4, 5). Besides, ROS can also trigger apoptosis via different mechanisms involving the p53, JAK/STAT, and tumour necrosis factor(TNF)-α-mediated pathways (6). Furthermore, the complement cascade is activated by ischaemic myocardial injury (7, 8) which also leads to neutrophil and monocyte recruitment into the ischaemic area (9). Whether apoptosis or necrosis contribute to cardiomyocyte cell death still remains a matter of debate. Some studies state that apoptosis only occurs during reperfusion, while other studies report apoptosis in the cardiomyocyte to start during the ischaemic process (10, 11). Nevertheless, apoptosis was shown to contribute to myocardial ischaemia/reperfusion (I/R) injury in animal models. In humans, apoptosis has been particularly demonstrated in the border zone of the infarcted area (12, 13).

The response of the myocardium to ischaemic injury has been described as cardiac hypertrophy, recognised by the enlargement of cardiomyocytes without increasing in number. Cardiomyocytes can enlarge either in thickness by laying down myofibrils in parallel, or in length by laying down new sarcomers to existing myofibrils. Furthermore, hypertrophy is recognised by proliferation of the connective tissue to maintain vascularity for the enlarged cardiomyocytes (14, 15). The second highest mortality rate due to CVD is caused by ischaemic stroke as it accounts for 32% of all cardiovascular related...
deaths. As in myocardial I/R injury, the inflammatory process plays a major role in the progress of I/R injury. Upon reperfusion, leukocytes are activated and are able to disrupt the blood brain barrier. This eventually leads to infiltration of leukocytes into the brain tissue releasing pro-inflammatory cytokines and ROS (16). Furthermore, the complement system is activated, resulting in the activation of a number of inflammatory mediators including C5 and the membrane attack complex (MAC) that leads to further leukocyte infiltration, the release of TNF-α, interleukin (IL)-1β, and interferon (IFN)-γ, and an increase in cell membrane permeability (17, 18). Whereas at first necrosis was considered the major form of cell death, apoptosis also serves a crucial role in cerebral I/R injury. This process of apoptosis occurs mainly within the neurons within the ischaemic penumbra (19). The apoptotic process starts with the release of cytochrome c from the mitochondria under the influence of ROS and is mediated by proapoptotic proteins. When cytochrome c is translocated to the cytosol, it activates a number of caspases, leading to DNA fragmentation and the activation of p53 (20). Furthermore, also astrocytes are thought to play a fundamental role in the pathogenesis of neuronal cell death (21).

Renal ischaemia is also of major importance as it accounts for 50% of acute renal failure (ARF) (22) and is a major contributing factor to graft rejection following kidney transplantation. The causes of ARF can be diverse and usually do not relate to acute thrombotic occlusion of renal arteries, although atherosclerosis of renal arteries is a contributor to chronic renal failure in conditions like hypertension and diabetes. ARF in graft rejection can, however, be caused by thrombotic and/or fibrous occlusion of the microvasculature. Upon acute renal ischaemia, the proximal tubule cells are the most affected and damage of these cells causes a reduction in glomerular filtration rate (GFR). A number of mechanisms seem to be involved in this process, indicating an important role for the actin cytoskeleton (23). First, ischaemia causes the shedding of cell debris into the tubular lumen resulting in cast formation, tubular obstruction, and increased tubular pressure (24, 25). Second, ischaemia results in opening of tight junctions and leakage of the glomerular filtrate into the blood impairing glomerular filtration (26). Third, ATP deficiency causes tubular cell swelling due to a disturbed sodium reabsorption leading to further cellular injury (27). In the past, ischaemic cell death was thought to be caused by necrosis. However, a number of apoptotic pathways appear to be involved in ischaemic cell death including mitochondrial non-caspase and caspase pathways (28). The process of inflammation, which results from cell death, plays an important role in renal I/R injury. Inflammation is also aggravated by the increase in vascular permeability, the recruitment of leukocytes into the kidney, and the up-regulation of several inflammatory mediators including IL-1, IL-6, IL-8 and TNF-α (29, 30).

Hepatic I/R injury is in most cases caused by hepatic surgery or transplantation and remains an important clinical problem as it is the cause of organ failure leading to higher numbers of graft rejection. Complex processes are involved in liver I/R injury and comprise of the formation of ROS, the activation of the complement system and cell adhesion molecules, the secretion of cytokines and chemokines including TNF-α, IL-1β, and IFN-γ, and the activation of endothelial cells, Kupffer cells and polymorphonuclear leukocytes (PMNs). These processes finally result in cellular swelling, the break-down of endothelial cells and the infiltration of PMNs leading to dysfunction of the liver cells (31). However, the exact chronology of these processes in the contribution to liver injury is not known.

In summary, in general I/R injury is recognised by a strong inflammatory response preceded or followed by apoptosis. The blood coagulation system is characterised by several potentially relevant interactions with the immune system. Hence, anti-coagulant treatment could be a tool to influence the complex cascade of I/R organ injury. Indeed, several pre-clinical reports have addressed these topics in specific organ I/R models and in this review we discuss the outcomes and potential significance of such animal studies.

**Crosstalk between coagulation and inflammation in I/R injury**

Coagulation and inflammation are two distinct processes, but interact at different levels. These interactions have already been studied for a long time and appear to play an important role in several pathologies including sepsis and disseminated intravascular coagulation (DIC) (32, 33). Also in I/R injury the crosstalk between inflammation and coagulation has been shown to be of major importance.

Upon I/R, monocytes and macrophages that expose tissue factor (TF) are recruited into the area of ischaemia. The cellular derived TF forms a complex with factor (F)VIIa, thereby activating the coagulation cascade, which eventually results in the formation of a fibrin network (34). Different procoagulant pathways can be inhibited by different inhibitors including activated protein C (APC), tissue factor pathway inhibitor (TFPI), and antithrombin (AT) (35–38).

Coagulation proteases mediate important effects in cell signalling through activation of G-protein coupled protease activated receptors (PARs). Four types of PARs have been identified and each is activated by a serine protease, which cleaves specific sites leading to the formation of a neo N-terminus beginning with a specific sequence functioning as a tethered ligand. The ligand binds to a second transmembrane loop of the receptor, triggering G-protein binding and intracellular signalling. Despite their similar mechanisms of activation, the different PARs are present on different cell types including endothelial cells, cardiomyocytes, and vascular smooth muscle cells (39) and trigger various cellular processes. PAR-1 is primarily a thrombin receptor but can also be activated by the TF/FVIIa complex, FXa, and APC although their affinity for PAR-1 is less than that of thrombin. PAR-1 activation by thrombin results in the expression of adhesion molecules on endothelial cells and the activation of monocytes and endothelial cells which release various cytokines and chemokines (40, 41). The role of PAR-1 in I/R injury has been revealed by Junge et al. They showed that mice lacking PAR-1 had a reduced infarct size after cerebral ischaemia.
compared to normal mice (42). Furthermore, PAR-1 plays a mediating role in cardiomyocyte hypertrophy and remodeling upon ischemia as PAR-1 deficiency reduced left ventricle dilatation and improved left ventricle function (43). In contrast to PAR-1, PAR-2 is not a thrombin receptor but can be activated by the TF/FVIIa complex (44), Fxa, and APC, although APC is most likely to signal through PAR-1 (45). Activation of PAR-2 by TF/FVIIa leads to an up-regulation of the inflammatory response in macrophages by the expression of cell adhesion molecules and production of ROS and an increase in leukocyte infiltration combined with increased expression levels of TNF-α and IL-1β (46). PAR-2 has a modulating effect on myocardial I/R injury in rats as activation of PAR-2 recovered myocardial function and decreased oxidation at reflow (47). A role for neither PAR-3 nor PAR-4 has been revealed in I/R injury.

Although PARs are believed to serve an important role in I/R injury, several anti-coagulant mechanisms are involved in inflammatory processes independent from these receptors. AT is shown to inhibit PGI2 release from the endothelium, thereby inhibiting the synthesis of TNF-α through the regulation of NF-κB (48). Furthermore, AT administration inhibited NO production with increased inflammatory response upon LPS challenge (49) and prevented leukocyte recruitment (50). APC also had direct anti-inflammatory effects through reduction of TNF-κB signalling in monocytes and through a reduced ability of inflammatory mediators to induce TF in leukocytes (51–53). Furthermore, thrombomodulin (TM) is known to reduce MAPK (mitogen-activated protein kinases) and NF-κB signalling (54). Finally, when thrombin is bound to TM, thrombin-activatable fibrinolysis inhibitor (TAFI) is activated which can inhibit complement factor C5a, contributing to a reduction in microvascular injury (55).

Anti-coagulants in ischaemia/reperfusion models

A large number of classic anticoagulants have been analysed in I/R injury models to reveal their protective properties. Heparin has been shown to have a cardioprotective function upon induction of I/R injury (56, 57) and heparin as well hirudin were shown to attenuate leukocyte adhesion upon ischemic muscle injury (58). Furthermore, hirudin was shown to be protective in a stroke model (59). Within the scope of this review a number of anticoagulants are selected on the basis of their pronounced cell protective properties in animal models of I/R injury. As these (natural) anticoagulants are used in animal models, the species specificity of these molecules remains an important matter of debate. For the anti-coagulant APC, species specificity has been demonstrated and was shown to be influenced by interaction with protein S (60, 61). Furthermore, the anti-coagulant TFPI exist in different alternatively spliced forms in different species, possibly contributing to the species specificity of TFPI (62, 63). Also, the association between mouse TF and human FVIIa was shown to be much weaker compared to human TF so this has to be taken into account in applications of human FVIIa in animal studies (64). Summarising, as these anticoagulant proteins all show strong species specificity, the effects of human variants within animal models need to be considered carefully. An overview of the use of anticoagulants in animal models of I/R injury is given in Table 1.

Activated protein C (APC)

Upon activation of coagulation, thrombin binds to TM activating protein C into APC at a relatively slow rate. However, the rate of

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Species</th>
<th>I/R model</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheng et al. (70)</td>
<td>Mouse APC</td>
<td>Mouse</td>
<td>Brain</td>
<td>Neuroprotective, anti-apoptotic</td>
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<tr>
<td>Shibata et al. (73)</td>
<td>Human APC</td>
<td>Mouse</td>
<td>Brain</td>
<td>Neuroprotective, anti-inflammatory</td>
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<tr>
<td>Zlokovic et al. (74)</td>
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<td>Hirose et al. (75)</td>
<td>Human APC</td>
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<td>Spinal cord</td>
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<tr>
<td>Dillon et al. (76)</td>
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<tr>
<td>Loubele et al. (79)</td>
<td>Mouse APC</td>
<td>Mouse</td>
<td>Heart</td>
<td>Reduction infarct size, anti-inflammatory, antiapoptotic</td>
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<tr>
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<td>Human FVIIia</td>
<td>Rabbit</td>
<td>Heart</td>
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</tr>
<tr>
<td>Loubele et al. (92)</td>
<td>Mouse ASIS</td>
<td>Mouse</td>
<td>Heart</td>
<td>Reduction infarct size, anti-inflammatory</td>
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<tr>
<td>Olanders et al. (93)</td>
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<tr>
<td>Erlich et al. (95)</td>
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<td>Heart</td>
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<tr>
<td>Koudsi et al. (96)</td>
<td>Human TFPI</td>
<td>Rabbit</td>
<td>Spinal cord</td>
<td>Improvement neurological function</td>
</tr>
<tr>
<td>Ushigome et al. (97)</td>
<td>Human TFPI</td>
<td>Rat</td>
<td>Kidney</td>
<td>Reduction necrosis</td>
</tr>
<tr>
<td>Schoots et al. (98)</td>
<td>Human AT</td>
<td>Rat</td>
<td>Intestine</td>
<td>Reduction histological changes, anti-inflammatory</td>
</tr>
<tr>
<td>Ozden et al. (99)</td>
<td>Human AT</td>
<td>Rat</td>
<td>Kidney</td>
<td>Reduction histological changes, anti-inflammatory</td>
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Table 1: Overview of the effects of several anticoagulants on I/R injury.
protein C activation is increased 20-fold in the presence of the endothelial protein C receptor (EPCR) (65, 66). APC together with protein S can exert its anticoagulant effects via the proteolytic inhibition of either FVa or FVIIa (67). Besides its role in anticoagulation, APC is also involved in cell signalling mechanisms via PAR-1 (40). PAR-1 on endothelial cells is cleaved by APC in an EPCR-dependent manner (68). In vitro studies revealed much of the cytoprotective and cell signalling properties of APC (45, 69–72). APC also showed protective effects in several ischaemia models. In an ischaemic stroke model, APC has been proven to have anti-inflammatory, antithrombotic, and neuroprotective effects as the administration of APC increased the average survival time and restored cerebral blood flow (70, 73, 74). Furthermore, APC has anti-inflammatory properties in a rat model of spinal cord I/R by inhibition of neutrophil activation (75) and in an acute skeletal muscle I/R model by reducing myeloperoxidase (MPO) content and improving electrical properties of skeletal muscle (76). Mizutani et al. demonstrated a reduction in I/R induced renal injury by APC as administration of APC led to an improved renal blood flow after I/R, to an increased vascular permeability, to reduction in fibrin degradation products in plasma, and reduced TNF-α, IL-8 and MPO plasma concentrations (77). However, in a different murine model, APC did not attenuate renal I/R damage and the protective effects of APC may well be depending on the exact model used (Loubele et al., unpublished data). Cheng et al. have shown an anti-apoptotic role for APC in vitro in brain endothelium via the inhibition of p53 and caspase signalling as well as in vivo in a focal ischaemic stroke model. Administration of APC reduced brain infarction volumes and brain oedema, requiring the presence of EPCR and PAR-1 (70). Furthermore, administration of APC reduced hepatic I/R injury as administration of APC improved histological findings of I/R injury, reduced neutrophil infiltration and the expression of adhesion molecules (78). APC was also shown to be protective in a myocardial I/R model via reduction of inflammation and apoptosis in a PAR-1-dependent manner (79).

As it is not clear yet whether the anticoagulant or the cell signalling functions of APC are responsible for the protective effect of APC, mutant forms of APC are being developed and tested in a number of animal studies. The results point to differences in the relative importance of the anticoagulant over the PAR mediated functions of APC, in relation to outcome. In a sepsis model, usage of a non-anticoagulant form of APC provided an equal protective effect compared to wild-type APC with regard to survival rates while the risk for bleeding is reduced (80). In an acute kidney injury model, however, both anticoagulant and the PAR-mediated signalling effects of APC offered protection against renal injury (81). In a model of ischaemic stroke where the anticoagulant activity of APC is undesirable given the substantial detrimental consequences of bleeding, the non-anticoagulant 3K3A-APC mutant was more effective in reducing cerebral damage than wild-type APC, without apparent increased risk for bleeding (82).

**Active site inhibited FVIIa (ASIS)**

Active site inhibited FVIIa (ASIS), or FVIIai, is a recombinant FVIIa which is blocked at its catalytic site by the tripeptide (Phe-Phe-Arg) chloromethyl ketone, thereby competitively inhibiting FVIIa binding to TF. ASIS has via inhibition of TF/FVIIa signalling a direct function on several cellular processes. Besides its role in thrombus formation, TF plays a role in the regulation of inflammation, migration and proliferation of vascular smooth muscle cells, and the formation of embryonic blood vessels, mostly via its interaction with FVII (83–85). A number of studies propose a role for the cytoplasmatic tail of TF in cell adhesion and migration (86, 87) but most of the cell signalling functions depend on the complex formation of TF with FVIIa. The binding of FVII or FVIIa to TF is Ca²⁺-dependent and triggers several cell signalling functions that result in regulation of apoptosis (88, 89). Most of these processes are regulated via PAR-2 as blocking PAR-2 also blocks TF/FVIIa regulated cell migration (90). During I/R injury, human recombinant FVIIai reduced infarct size in a rabbit myocardial I/R model via inhibition of the procoagulant (i.e. generation of thrombin) activity of TF (91). Furthermore, ASIS administration was shown to be protective in a mouse model of myocardial I/R via inhibition of inflammation, regulated via an NF-kB-mediated mechanism (92). In a rat model of intestinal I/R, FVIIai displayed anti-inflammatory properties. Intestinal ischaemia was induced for 40 minutes (min) followed by a 6 hour (h) reperfusion period. Pretreatment with FVIIai in this I/R model reduced the permeability of the endothelial barrier, and lowered MPO activity and matrix inflammatory protein (MIP)-2 levels (93). An anti-apoptotic role for FVIIai has not yet been revealed. Experiments performed in mice either expressing 1% of the human TF levels (low-TF mice) or mice lacking PAR-1, however, demonstrated that these mice were protected from renal failure after 25 min of renal ischaemia and varying periods of reperfusion. Furthermore, these mice showed reduced mortality rates, neutrophil accumulation, and chemokine levels indicating a role for TF in I/R injury regulated by PAR-1 (94). Erlich et al. showed a positive effect of an anti-rabbit TF monoclonal antibody on infarct size in a myocardial I/R model most likely through attenuation of inflammation. A rabbit coronary ligation model was used in which administration of a TF antibody reduced infarct size and decreased both chemokine expression and leukocyte infiltration (95). The consequences of competition of ASIS with binding of FVIIa to TF on cell signalling properties are not extensively known.

**TFPI**

TFPI is a serine protease inhibitor of the TF/FVIIa complex and is the only natural regulator of the TF-dependent pathway of coagulation known so far. TFPI was demonstrated to have some protective effects in ischaemic injury but the underlying mechanisms have not been extensively characterised yet. In rabbits, TFPI appears to positively affect neurologic function repair after I/R injury.
of the spinal cord. The infrarenal aorta was occluded via a snare occlusion device for 21 min followed by a three day reperfusion period. The number of animals retaining neurological function after TFPI treatment was significantly higher compared to the placebo treated animals suggesting a role for TFPI in preventing I/R injury (96). In a rat model TFPI also attenuated renal I/R injury; renal ischaemia was induced by clamping both left renal vein and artery. Animals receiving TFPI had a smaller necrotic area compared to placebo treated animals, suggesting a role for TFPI in modulation I/R injury (97).

**Antithrombin (AT)**

Another natural inhibitor of coagulation is AT which functions as a general protease inhibitor. Besides thrombin, AT inhibits a large number of intrinsic route serine proteases including FIXa, FXa, and FXIa. AT has two major reaction sites, one necessary for anticoagulant properties in animal models of I/R injury. Schoots et al. used a rat superior mesenteric artery I/R model in which vessels were subjected to 20 or 40 min of ischaemia followed by a 3 h reperfusion period. Administration of AT caused a decrease in I/R-induced intestinal dysfunction, histological changes, thrombin generation, fibrin degradation products, and fibrin deposition compared with placebo treated animals. A reduction in the inflammatory response was noted as was demonstrated by a reduction in IL-6 plasma levels compared to control animals (98). The anti-inflammatory properties of AT have been assessed in a rat model of renal I/R. After a 60 min ischaemia and 24 h reperfusion period, the administration of AT lead to decreased creatinine and MPO activity levels and there was less histopathological damage in the AT group compared to the control group. Accumulation of inflammatory mediators like neutrophils was inhibited by AT treatment, suggesting an anti-inflammatory effect of AT in renal I/R injury, however, leaving open the question of which intermediate proteases were primarily involved (99).

An overview of the different anti-coagulants with their effects and known signalling pathway is given in Figure 1.

**Clinical use of anti-coagulants**

The use of anticoagulant agents as anti-inflammatory therapeutics has already been extensively investigated in sepsis. AT, APC and TFPI each have been shown to have strong beneficial effects on mortality in baboon sepsis models (100–102). All three anti-coagulants were subsequently tested in large clinical trials of sepsis patients. Of these compounds only APC reduced the relative death risk in sepsis patients with 19.4%; however, at the price of an increased bleeding risk (103). Administration of ASIS in endotoxin-induced coagulation in humans, revealed a blockage of thrombin and fibrin generation, but no apparent anti-inflammatory effects (104). The anticoagulants AT and TFPI failed to show a protective effect in clinical trials of patients with severe sepsis, while their use was associated with an increased bleeding risk (105, 106). The large discrepancy between large animal and human studies may be due to a range of factors including the absence of (co-)morbidities in laboratory animals, as compared to genetic and phenotypic heterogeneity, concurrent use of medication, heterogeneity in the course of sepsis in humans, whereas this is carefully timed in animals and so on. The same problems will no doubt emerge when predicting human responses to anticoagulant agents in I/R injury settings on the basis of findings in animal models. Despite the promising results of the use of anticoagulants as protection against I/R injury in animal models, none of these have so far been tested for their anti-inflammatory or anti-apoptotic characteristics in human studies. However, given the extensive interactions between coagulation and inflammation in experimental models of complex disease, including I/R injury, the therapeutic potential of (mutant forms of) anticoagulant proteins with cell signalling properties definitely deserves more attention.

**References**


